REFERENCES.

BADENSKI, G., AND BRUCKNER, I.—(1938) *C.R. Soc. Biol.*, **129**, 406 and 408.
BURNET, F. M.—(1936*a*) *Aust. J. exp. Biol.*, **14**, 241 and 247.—(1936*b*) *Med. Rec. Counc. Spec. Rept. No.* 220.—(1936*c*) *Brit. J. exp. Path.*, **17**, 282.
BURNET, F. M., AND FERRY, J. D.—(1934) *Ibid.*, **15**, 56.
BURNET, F. M., KEOGH, E. V., AND LUSH, DORA.—(1937) *Aust. J. exp. Biol.*, **15**, (Supplement), 231.
GLOVER, R. E.—(1938) *Vet. Rec.*, **50**, 377.
MESROBEANU, I.—(1938) *C.R. Soc. Biol.*, **127**, 1183.
SHOPE, R. E.—(1933) *J. exp. Med.*, **57**, 925.
TRAUB, E.—(1933) *Ibid.*, **58**, 663.
WOODRUFF, A. M., AND GOODPASTURE, E. W.—(1931) *Amer. J. Path.*, **7**, 209.

THE IMMUNOLOGICAL RELATIONSHIP OF THE VIRUS OF SPONTANEOUS COWPOX TO VACCINIA VIRUS.

A. W. DOWNIE.

From the Bacteriological Department of the London Hospital and Medical College, London, E. 1.

Received for publication February 22nd, 1939.

THE observations of Jenner (1798) indicated the close immunological relationship between the virus of cowpox and that of smallpox, but it is evident from the publications of Crookshank (1889), Cory (1898) and Copeman (1899) that, of the so-called " vaccinia " strains in current use in various parts of the world towards the end of last century, many had been derived by passing human smallpox through animals. The more recent literature on the transformation of variola to vaccinia has recently been reviewed and discussed in relation to his own observations by Horgan (1938).

Although the terms " vaccinia " and " cowpox " are frequently used synonymously, it seems doubtful whether the viruses concerned should be regarded as identical, yet in much of the earlier work on the protective power of " vaccinia " against the virus of smallpox in man or monkeys (Copeman) such identity is assumed. The more recent observations of Green (1908), Reece (1921), Van Heelsbergen (1922), Frenkel (1930), Glover (1937) and others have shown that the virus of spontaneous cowpox and laboratory strains of vaccinia produce effective immunity against each other in animals.

While there doubtless exists a close relationship between these viruses, the examination of convalescent sera from three men who had contracted infection from the spontaneous disease in cattle (Davies, Janes and Downie, 1938)

suggested that the virus isolated from one of the men was not identical with a strain of vaccinia in current use. A comparative study of the lesions produced experimentally by three strains derived from the natural disease in the cow and three strains of vaccinia also indicated differences which have been described elsewhere (Downie, 1939). The methods which have recently been developed by Craigie (1932) have provided a means whereby a more accurate knowledge of the antigens of vaccinia virus and the antibodies in antivaccinial serum has been gained (Craigie and Wishart, 1934*a* and 1936 ; Parker and Rivers, 1935 ; Salaman, 1937). By utilizing the methods developed by these workers for the preparation of relatively pure suspensions of virus and the carrying out of serological tests, an attempt has been made to determine the immunological relationship between two strains of vaccinia virus and two strains of the virus of spontaneous cowpox. The results of these investigations are recorded in this paper.

MATERIAL AND METHODS.

Strains of Virus.

Cowpox virus, strain B, was isolated from the lesions on the hands of a cowman on a farm in Sussex in 1937, and had been passed by pad inoculation through two guinea-pigs, and thereafter by intradermal inoculation or by scarification in the skin of rabbits.

Cowpox virus, strain G, was isolated in 1937 from scabs derived from typical lesions on a cow by Mr. R. E. Glover, of the National Institute for Medical Research, Millhill. It was passed intradermally through 3 rabbits, then by intratesticular inoculation through two rabbits, and thereafter by intradermal or cutaneous inoculation on these animals.

Vaccinia virus, strain S.—This was the stock Lister Institute strain which had been passed repeatedly by cutaneous inoculation in rabbits, and was used by Dr. M. H. Salaman in his work on the combining properties of vaccinia virus with the antibodies demonstrable in antivaccinial serum (Salaman, 1937). This strain has been in use at the Lister Institute for many years, but it is uncertain whether it was originally obtained from spontaneous cowpox or from variola in man.

Vaccinia virus, strain A, was derived by Dr. C. R. Amies from smallpox material obtained in 1932, and had been repeatedly passed in rabbits by cutaneous inoculation.

An account of the lesions produced by these four strains of virus on the chorio-allantoic membranes of developing hens' eggs, in guinea-pigs and in rabbits has been reported elsewhere. In the experiments recorded below all immunity tests were made in rabbits. In some of the earlier experiments the supernatant fluid from infected egg-membranes or from the ground-up lesions which had resulted from intradermal injection in the rabbit was the infective material, but in all later experiments elementary body suspension prepared from cutaneous infection in rabbits has been used except in one instance, when a similar suspension was made from infected egg-membranes.

Preparation of Elementary Body Suspensions and Soluble Specific Substance.

The method of Craigie (1932) as modified by Salaman (1937) was used throughout. Rabbits were inoculated on the shaved and lightly scarified skin of the back with elementary body suspension and were killed and exsanguinated on the 3rd or 4th day afterwards. Elementary body suspension and crude Seitz filtrates containing the soluble specific substance were prepared according to the technique described in detail by Salaman using M/250 citric acid + disodium phosphate buffer pH 7·1 for successive washings and final suspension. It may be observed that while the titre of soluble specific substance obtained from the four strains of virus was approximately the same as indicated by complement-fixation tests with appropriate immune sera, the yield of thrice washed elementary bodies was usually greater with vaccinia virus, strain S, than with the other three strains. Examination of the suspensions under dark-ground illumination and by stained films indicated that they were relatively pure, although with the three latter strains a certain amount of ill-defined, finely granular material less refractile than the elementary bodies was frequently present in the final suspensions. The number of bacteria in the suspensions was always estimated by cultures made in blood-agar, and these showed counts varying from three to several hundred colonies from 0·1 c.c. When suspensions were not required for immediate use they were stored at a temperature of 0° to 4° C. under ether.

Preparation of Antisera.

In certain experiments the sera from rabbits which had received multiple intradermal injections of virus 2 to 4 weeks previously were used, but the sera for absorption experiments were obtained from animals which had received two to four further injections of virulent elementary body suspension. These injections were made intraperitoneally or intravenously at intervals of 7 to 14 days, and the rabbits were bled 6 to 8 days after injection. The sera were heated at 56° C. for 40 minutes and were stored without preservative. In general it has been found that good antisera are more easily obtained against the cowpox than against the vaccinia strains. Sera from rabbits which had been infected intradermally with cowpox virus two or three weeks previously showed good neutralizing power and a good titre of complement-fixing antibody whereas, with the vaccinia strains, hyperimmunization was usually necessary to obtain sera with a satisfactory antibody content.

Agglutination Tests with Elementary Body Suspensions.

The method used was that described by Craigie and Wishart (1934*a*). Dilutions of serum were made in 0·85 per cent. NaCl containing M/250 citric acid + disodium phosphate, pH 7·1. Stock suspensions of elementary bodies were diluted before use in M/250 buffer to give a turbidity comparable to that of bacterial suspensions suitable for agglutination. Equal volumes (0·3 c.c.) of serum dilutions and suspension were mixed in small tubes, which were then incubated under cover in a water-bath at 50° C. The results were read after

3 hours and again after 18 to 20 hours. Normal rabbit serum was always included for control purposes.

Complement-fixation Tests.

The complement-fixation reaction with soluble precipitable substance contained in Seitz filtrates of vaccinia skin pulp and anti-vaccinial sera has been found by Craigie and Wishart (1934*b*) to give results comparable to precipitation tests. In preliminary tests made with the Seitz filtrates prepared from the cowpox viruses used in this work, the complement-fixation reaction was found to be more sensitive and to give sharper results than the precipitation test, and in consequence has been used extensively for the titration of soluble specific substance or antibody in immune sera.

To 0·2 c.c. quantities of a suitable dilution of serum or filtrate 0·2 c.c. of guinea-pig serum diluted to contain 3 M.D.H. of complement and 0·2 c.c. of dilutions of filtrate or serum were added. The tubes were kept at room temperature for 4 hours before the addition of 0·4 c.c. of sensitized cells (equal volumes of 5 per cent. sheep cells and amboceptor containing 5 M.D.H.). The tubes were then placed in a water-bath at 37° C. for 20 to 30 minutes and afterwards at room temperature. The results were read after 1 hour, and again after the racks had been left on the bench overnight. Suitable controls of antigen and serum were included in each test. In preliminary tests the findings of Craigie and Wishart in respect of the more complete fixation obtained at 4° C. for 16 hours were confirmed, but for most purposes fixation for 4 hours at room temperature gave satisfactory results and has been used throughout.

Absorption of Sera.

In attempting to determine immunological differences between the cowpox and vaccinia strains, cross-absorption of hyperimmune sera with elementary body suspensions has been used. It has been noted by Salaman (1937) that more complete absorption of an antivaccinial serum is obtained when the absorption is carried out with three successive fractions than when a single absorption with the same dose of elementary bodies is made. For the purpose of the present work complete absorption of sera was not considered necessary, and it has been found that, with most sera, adequate absorption of homologous antibody could be obtained by using the yield of elementary bodies from one rabbit for 1·0 c.c. of immune serum. Prior to absorption of sera the washed elementary body suspension was spun out in the angle centrifuge, and the deposit taken up in 2–3 c.c. of M/250 citric acid + disodium phosphate buffer. To 1·0 c.c. of immune serum one-third of the elementary body suspension was added, and the volume made up to 3·0 c.c. with distilled water and 8·5 per cent. salt solution to give a final concentration of 0·85 per cent. The mixture was allowed to stand at room temperature for 1 hour and then placed at 0°–4° C. overnight. Next day the mixture was spun in the angle centrifuge and the clear supernatant pipetted off. The second fraction of elementary body suspension was added, and the volume made up to 4 c.c. with distilled water and 8·5 per cent. as before, and absorption allowed to proceed as in the first instance. The third absorption was similarly made and the final supernatant

of 5·0 c.c., representing a 1 in 5 dilution of the original serum, was heated at a temperature of 56° C. for 1 hour to destroy any live virus which might have been present. Control unabsorbed sera were always subjected to the same temperature as absorbed sera. The absorbed sera were tested for residual agglutinins, complement-fixing and neutralizing antibody. In certain of the earlier experiments the mixtures were placed in a water-bath at 50° C. for 2 hours during absorption, but equally good absorption seemed to occur when the mixtures were kept at 0°–4° C. overnight, and this procedure was followed in most of the experiments recorded below.

Neutralization Tests.

Equal volumes of 1/5 dilutions of absorbed or unabsorbed sera were mixed with falling 10-fold dilutions made in broth of elementary body suspension of the virus under examination. After the tubes had been kept at room temperature for 1 hour 0·2 c.c. of each mixture was injected intradermally into normal rabbits. All sera to be compared were titrated on the same animals, two rabbits being used for the injection of the same serum-virus mixtures in each test. The readings recorded in the following tables were made on the 4th or 5th day after injection.

Housing of Animals.

Owing to the limited accommodation available it was impossible to keep animals infected with the four viruses in separate animal houses. Animals infected with cowpox strain B and cowpox strain G were kept in different rooms in the same building, while the rabbits infected with the vaccinia strains were kept in one room in another building several hundred yards from the first. The experiments with the two vaccinia strains were so arranged that animals used for preparation of elementary body suspensions of the two strains were not kept in the one animal house at the same time. In certain experiments made to test resistance of immunized animals and involving the injection of different strains of virus, these animals were removed when necessary to the building used for the vaccinia work immediately before the test injections were made. Animals infected with the vaccinia strains were never kept in the building which housed the animals used for the cowpox viruses. These precautions were considered necessary in view of the ease with which vaccinia is reported to spread among rabbits kept in the same room.

EXPERIMENTAL.

Cross-immunity in Rabbits Recovered from Infection with Cowpox or Vaccinia Virus.

Many of the animals used for titration of cowpox strain B and vaccinia strain S were later tested for resistance to intradermal injection of these two strains. Table I shows the results of such tests on two rabbits which recovered from infection with vaccinia S (V113 and V267), and two which had recovered from infection with cowpox B (B34 and B44). The first three animals shown

TABLE I.—*Cross immunity Tests in Animals Recovered from Infection with Cowpox Virus, Strain B, and Vaccinia, Strain S.*

Rabbit number.	Previous infection with—	Interval between infection and test.	Tested by intradermal injection of—											
			Vaccinia, strain S.						Cowpox, strain B.					
			Dilutions of virus.											
			10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.
V. 113	Vaccinia S	21 days	+	+	+	±	−	0	+	+	+	±	−	0
V. 267	,,	14 ,,	+	+	+	−	−	0	+	±	±	−	−	0
Normal rabbit	..	..	++	++	++	++	+	±	++	++	++	++	+	±
B. 34	Cowpox B	30 ,,	+	+	+	±	±	0	±	±	±	±	−	0
B. 44	,,	15 ,,	+	+	±	±	−	0	+	±	−	−	−	0
Normal rabbit	..	..	++	++	++	+	+	±	++	++	++	++	+	±

++ Signifies a lesion of average diameter 15 mm. or more.
\+ ,, ,, ,, ,, 7–15 mm.
± ,, a tiny papule less than 7 mm. in diameter.
− ,, complete absence of reaction.
0 ,, not tested.

TABLE II.—*Cross-immunity Tests on Hyperimmunized Rabbits.*

Rabbit number.	Immunized with—	Interval between last immunizing injection and test.	Tested with—	Dilutions of virus injected intradermally.							
				10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	10^{-8}.
B. 82	Cowpox B	8 weeks	Cowpox B	+	±	−	−	−	−	..	..
			,, G	+	+	−	−	−	−	..	..
			Vaccinia S	+	+	±	−	−	−	..	..
B. 83	,, B	8 ,,	Cowpox B	+	+	±	−	−	−	..	..
			,, G	+	+	+	±	−	−	..	..
			Vaccinia S	+	+	+	+	±	−	..	..
G. 101	,, G	11 days	Cowpox B	+	+	±	−	−	−	..	..
			,, G	+	+	+	−	−	−	..	..
			Vaccinia S	+	+	+	+	−	−	..	..
G. 105	,, G	11 ,,	Cowpox B	+	−	−	−	−	−	..	..
			,, G	+	−	−	−	−	−	..	..
			Vaccinia S	+	+	−	−	−	−	..	..
139	Normal animal		Cowpox B	..	..	++	++	++	++	+	−
			,, G	..	..	++	++	++	++	±	−
			Vaccinia S	..	..	++	++	++	++	+	±
140	,, ,,		Cowpox B	..	..	++	++	++	+	−	−
			,, G	..	..	++	++	++	+	±	−
			Vaccinia S	..	..	++	++	++	+	±	−
V. 136	Vaccinia S	21 days	Cowpox B	+	+	±	−	−	−	..	..
			,, G	+	±	−	−	−	−	..	..
			Vaccinia S	+	+	±	−	−	−	..	..
196	Normal animal		Cowpox B	..	..	++	++	++	++	±	−
			,, G	..	..	++	++	+	±	−	−
			Vaccinia S	..	..	++	++	+	±	−	−
V. 273	Vaccinia A	10 ,,	Cowpox B	−	−	−	−	−	−	..	..
			Vaccinia S	++	++	+	−	−	−	..	..
			,, A	+	+	±	−	−	−	..	..
V. 276	,, A	10 ,,	Cowpox B	+	+	−	−	−	−	..	..
			Vaccinia S	++	++	+	+	−	−	..	..
			,, A	++	+	+	−	−	−	..	..
286	Normal animal		Cowpox B	..	..	++	++	++	±	−	−
			Vaccinia S	..	..	++	++	++	++	+	±
			,, A	..	..	++	++	++	+	±	−
287	,, ,,		Cowpox B	..	..	++	+	+	−	−	−
			Vaccinia S	..	..	++	++	++	++	+	−
			,, A	..	..	++	++	+	±	−	−

The signs have the same significance as in Table I.

in the table were tested 10 days before the other three. In the first test the cowpox material used for infection was the supernatant fluid from a suspension of tissue obtained from a 5-day intradermal lesion, while for the test with cowpox B in the other three animals culture virus from the 20th passage on eggs was used. The same elementary body suspension of vaccinia virus, prepared from infected rabbit-skin pulp, was used in both tests.

The results suggest that the rabbits recovered from infection with cowpox were rather more resistant to this strain than to vaccinia virus. The two groups of animals are obviously not strictly comparable, however, and it is evident that the rabbits which had recovered from infection with either of the two viruses showed considerable resistance to both.

Rabbits which had been used for the preparation of hyperimmune sera were also tested for resistance to infection with cowpox and vaccinia viruses. In these tests elementary body suspensions were used for the intradermal injections. All such suspensions were prepared from rabbit-skin lesions except in the last four animals, for which the elementary body suspension of cowpox strain B was prepared from infected egg-membranes of the 69th passage. The results of the tests on these hyperimmunized animals are shown in Table II.

The first six animals were injected on the same day with the same dilutions of elementary body suspensions; rabbits V136 and 196 were tested ou a later date, while the last four animals were tested together still later. Although there may have been a slight variation in the infective titre of the virus suspensions used in the three groups of animals, as indicated by the lesions produced in the control normal rabbits, it is obvious that hyperimmunization with living virus of any of the four strains induces a high degree of resistance to both cowpox and vaccinia.

Neutralization Tests with Convalescent Sera.

Various sera obtained from rabbits from 18 to 24 days after intradermal titration of strains cowpox B and vaccinia S were tested for neutralization against these two viruses. All sera obtained from cowpox convalescent rabbits showed good neutralization, but the sera of rabbits recovered from vaccinial infection were more variable in this respect. One weak antivaccinial serum showed some neutralizing power against vaccinia S virus, but little or none against cowpox B. The antivaccinial serum used for the experiments on Table III was collected from a rabbit 21 days after intradermal titration of strain S and showed a good neutralizing titre. The anticowpox serum was obtained 19 days after intradermal titration of cowpox strain B. The sera had been inactivated and were added, without dilution, to 10-fold dilutions of virus in preparing the mixtures for intradermal injection.

The results shown in Table III indicate that the convalescent sera from rabbits previously infected with either strain of virus neutralizes both. However, certain weak antivaccinial sera seemed to possess little or no neutralizing antibody for cowpox virus, and tests with the sera of three cowmen who had recovered from infection with cowpox virus showed considerable neutralizing antibody against this virus, but little against vaccinia virus, strain S (Davies,

TABLE III.—*Neutralization of Cowpox and Vaccinia Viruses with Rabbit Convalescent Sera.*

Sera in mixtures.	Rabbit number.	Dilutions of virus in mixtures with undiluted sera. Cowpox B. 10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	Rabbit number.	Vaccinia S. 10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.
Anticowpox B	45	—	—	—	—	—	O	37	±	—	—	—	—	O
Antivaccinial S	45	—	—	—	—	—	O	37	+	—	—	—	—	O
Normal rabbit	45	++	++	++	++	+	±	37	++	++	++	+	+	—
Anticowpox B	46	—	—	—	—	—	O	38	±	—	—	—	—	O
Antivaccinial S	46	—	—	—	—	—	O	38	+	±	—	—	—	O
Normal rabbit	46	++	++	++	++	+	±	38	++	++	++	++	+	±

The signs have the same significance as in Table I.

TABLE IV.—*Complement-fixation Tests with the Antisera to Two Cowpox Strains Unabsorbed and After Absorption with Elementary Bodies of the Two Strains.*

Sera.	Absorbed with E.Bs. of strain—	Antigens. Cowpox B, Seitz filtrate. Serum dilutions. 1/20.	1/40.	1/80.	1/160.	Cowpox G, Seitz filtrate. 1/20.	1/40.	1/80.	1/160.	No antigen serum controls. 1/20.	1/40.
Anti-B	Cowpox B	++++	+++	+	—	++++	++	—	—	—	—
	,, G	++++	+++	+	—	++++	+	—	—	—	—
	nil	++++	++++	++++	++	++++	++++	+++	+	—	..
Anti-G	Cowpox B	++++	+++	—	—	++++	++	—	—	+	—
	,, G	+++	++	—	—	+++	—	—	—	—	—
	nil	++++	++++	++++	+++	++++	++++	++++	++	—	..
Normal rabbit	*nil*	±	—	..	..	—	—	..	..	..	..

E.Bs. = Elementary bodies.
++++ signifies no hæmolysis, complete fixation.
— signifies complete hæmolysis, no fixation.
+++, ++, +, ±, ∓ increasing degrees of hæmolysis.

Janes and Downie, 1938). In view of these findings and the differences observed in the histology of the lesions produced experimentally by cowpox and vaccinia strains (Downie, 1939), it seemed possible that antigenic differences between these virus strains might be brought out by cross-absorption tests with hyperimmune sera. The results of such tests are recorded in the next section.

Cross-absorption Tests with Hyperimmune Sera.

Comparison of the Two Cowpox Strains.

The two cowpox strains produced identical lesions on animals and on the chorio-allantoic membranes of hens' eggs, and infection experiments on recovered animals failed to indicate immunological differences. However, they had been isolated in different parts of the country, and cross-absorption of hyperimmune sera with elementary body suspension was carried out to determine whether there were minor serological differences. The result of the tests with these two strains will serve to indicate the value of the methods used in the further examination of the cowpox and vaccinia strains. The absorption of each of the two sera by the two viruses was carried out under the same conditions at the same time. In each instance the first and second fractions of the total absorbing dose of elementary bodies were completely agglutinated after contact with the serum overnight in the ice-chest, while the third fraction was only partially agglutinated and the supernatant fluid remained very turbid. The four samples of absorbed serum obtained by centrifugation for 1 hour in the angle centrifuge represented a dilution of 1/5 of the original sera, and were heated for 1 hour at 56° C. together with the unabsorbed sera and normal rabbit serum similarly diluted. The seven samples of serum were then used for complement-fixation, agglutination and neutralization tests. The results are shown in Tables IV, V and VI. Four rabbits were used for the neutralization tests, the seven sera being tested against each virus in duplicate ; Table VI shows the readings from one rabbit of each pair.

From the results of the complement-fixation tests it is apparent that although absorption of the sera is not complete, the reduction in titre of each serum effected by the two strains of virus is approximately the same. The same relationship holds in the agglutination tests, although in this case the absorption of antiserum to cowpox G would appear to be almost complete. This difference in the results of complement-fixation and agglutination tests with absorbed sera has been observed throughout this work, and illustrates the greater delicacy of the complement-fixation test as a measure of residual antibody in absorbed sera. It has been shown by Craigie (1932), and Craigie and Wishart (1936), that the antigens in Seitz filtrates of crude vaccinia suspensions responsible for precipitation with antivaccinial serum are also concerned with the agglutination of elementary bodies by the same serum, and their work on complement-fixation (Craigie and Wishart, 1934*b*) suggests that here also the same antigens are concerned in the reaction with immune serum. Observations made in the course of the present work with cowpox

TABLE V.—*Agglutination Tests with the Sera shown in Table IV.*

Sera.	Absorbed with E.Bs. of strain—	E.B. suspensions used. Cowpox B. Serum dilutions. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	Cowpox G. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.
Anti-B	Cowpox B	++	+	±	—	—	—	+	+	—	—	—	—
	,, G	++	+	±	—	—	—	+	±	—	—	—	—
	nil	+++	+++	+++	+++	+++	+	+++	+++	+++	+++	++	±
Anti-G	Cowpox B	+	—	—	—	—	—	±	—	—	—	—	—
	,, G	±	—	—	—	—	—	—	—	—	—	—	—
	nil	+++	+++	+++	+++	++	+	+++	+++	++	++	+	±
Normal rabbit	*nil*	—	—	—	..	..	..	—	—	—	..	..	..

+++ = Complete agglutination and sedimentation.
++ = Agglutination with formation of coarse granules.
+ = Formation of granules easily visible with naked eye.
± = Formation of fine granules visible with the hand lens.
— = No agglutination.

TABLE VI.—*Neutralization Tests with the Sera shown in Table IV.*

Sera in mixtures.	Sera absorbed with E.Bs. of strain—	Virus in mixture. Cowpox B. Dilutions of virus added to 1/5 dilution of sera. 10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	Cowpox G. 10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	Controls (serum alone).
Anti-B	Cowpox B	20	16	17	7	—	..	15	15	10	5	—	..	--
	,, G	20	17	19	13	—	..	19	17	14	6	—	..	—
	nil	7	6	—	—	—	..	—	—	—	—	—	..	..
Anti-G	Cowpox B	24	24	26	22	11	..	21	20	17	12	4	..	—
	,, G	26	25	24	18	9	..	21	17	14	10	7	..	—
	nil	7	5	—	—	—	..	—	—	—	—	—	..	..
Normal rabbit	*nil*	26	26	28	24	14	5	21	21	19	16	9	3	..

Figures indicate the average diameter of the lesions in millimetres.

virus and its antiserum have yielded no evidence to suggest that complement-fixation tests with Seitz filtrates and agglutination of elementary bodies are due to different antibodies in cowpox antisera.

The results of the neutralization test shown on Table VI are similar to those obtained with the other two tests. The amount of elementary body suspension used for absorption of the two sera was the same, and the more complete exhaustion of the antiserum to cowpox G with both suspensions may have been due to the smaller amount or possibly the greater avidity of the antibody in the unabsorbed serum. Nevertheless the results of the tests with the absorbed sera give no indication of antigenic difference between these two cowpox strains.

It should be noted that in carrying out the neutralization tests the serum concentration is kept constant in the mixtures with dilutions of virus, which is the reverse of the procedure used in the agglutination and complement tests, so that exact parallelism in the results of neutralization and the other two tests with absorbed serum is hardly to be expected. There is some evidence from the work of Parker and Rivers (1936) and Salaman (1937) that neutralization of virus and precipitation with Seitz filtrates of vaccinia may not be due to the same antibody in antivaccinial serum. Nevertheless the active elementary bodies used for absorption of immune sera in the present experiments presumably contain all the antigens concerned in the reactions, and a similar reduction in titre of antibody in absorbed sera has been shown by the three methods of testing.

Comparison of the Two Vaccinia Strains.

In view of the history of the two vaccinia strains, one of which (strain A) was known to have been derived from human smallpox material, they were compared by the method of serum absorption as used for the cowpox strains. Strain A had not recently been passed so frequently by cutaneous inoculation of rabbits, and in consequence the amount of elementary body suspension recovered from each rabbit was less than in the case of strain S. The absorption of the two antisera was carried out in the same way as with the cowpox antisera, but unfortunately part of the antiserum to strain S which had been absorbed with elementary bodies of the same strain was lost during the final centrifugation. The part that remained was used for neutralization tests. The results of the examination of these antivaccinial sera are shown in Tables VII, VIII and IX.

It will be seen from the results of the tests with the unabsorbed sera shown in Tables VII and VIII that the antiserum to strain S was slightly stronger than that to strain A, and the elementary body suspension of strain S was more agglutinable than that of strain A, but neither the tests with unabsorbed nor with the absorbed sera indicate qualitative differences in the antigens of the elementary bodies of these two strains.

Comparison of the Cowpox Strains and Vaccinia, Strain S.

The relationship of the two cowpox strains to vaccinia virus strain S have been investigated by the methods of absorption of hyperimmune sera shown

TABLE VII.—*Complement-fixation Tests with the Antisera to Two Vaccinial Strains Unabsorbed and After Absorption with Elementary Bodies of the Two Strains.*

Sera.	Sera absorbed with E.Bs. of strain—	Antigens. Vaccinia S. Seitz filtrate. Serum dilutions. 1/20.	1/40.	1/80.	1/160.	1/320.	Vaccinia A. Seitz filtrate. Serum dilutions. 1/20.	1/40.	1/80.	1/160.	1/320.	Serum controls. 1/20.	1/40.
Anti-S	Vaccinia A	++++	+	—	—	—	++++	++	±	—	—	+	—
	nil	++++	++++	++++	++	±	++++	++++	++++	+++	±	—	—
Anti-A	Vaccinia S	++	—	—	—	—	+++	+	—	—	—	—	—
	,, A	+++	±	—	—	—	++++	++	—	—	—	—	—
	nil	++++	+++	+++	±	—	++++	++++	++++	++	—	—	—
Normal rabbit	*nil*	—	—	—	..	..	—	—	—	..	..	..	..

Notations as in Table IV.

TABLE VIII.—*Agglutination Tests with the Antisera shown in Table VII.*

Sera.	Sera absorbed with E.Bs. of strain—	E.B. suspensions. Vaccinia S. Serum dilutions. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	Vaccinia A. Serum dilutions. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.
Anti-S	Vaccinia A	±	±	—	—	—	—	—	—	—	—	—	—
	nil	+++	+++	+++	+++	++	+	+++	+++	++	++	+	±
Anti-A	Vaccinia S	—	—	—	—	—	—	±	—	—	—	—	—
	,, A	±	±	—	—	—	—	±	—	—	—	—	—
	nil	+++	+++	+++	++	+	±	+++	++	++	+	±	—
Normal rabbit	*nil*	—	—	—	..	..	..	—	—	—	..	..	..

Notations as in Table V.

TABLE IX.—*Neutralization Tests with Antisera to Two Vaccinia Strains Unabsorbed and After Absorption with Elementary Bodies of the Two Strains.*

Sera.	Sera absorbed with E.Bs. of strain—	Virus in mixtures. Vaccinia S. Dilutions of virus added to 1/5 dilution of sera. 10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	Vaccinia A. 10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	Serum alone.
Anti-S	Vaccinia S	17	10	7	—	—	17	13	9	—	—	—
	,, A	13	9	5	—	—	14	10	—	—	—	—
	nil	4	—	—	—	—	—	—	—	—	—	..
Anti-A	Vaccinia S	14	15	14	7	—	11	8	4	—	—	—
	,, A	15	14	13	6	—	8	7	—	—	—	—
	nil	—	5	—	—	—	—	—	—	—	—	..
Normal rabbit	*nil*	18	19	17	13	4	17	20	18	13	—	..

Notations as in Table VI.

TABLE X.—*Complement-fixation Tests with the Antisera to Cowpox G and Vaccinia S Unabsorbed and After Absorption with the Elementary Bodies of the Two Viruses.*

Anti-sera.	Sera absorbed with E.Bs. of strain—	Antigens. Cowpox G, Seitz filtrate. Serum dilutions. 1/20.	1/40.	1/80.	1/160.	1/320.	Vaccinia S, Seitz filtrate. 1/20.	1/40.	1/80.	1/160.	1/320.	Serum controls. 1/20.	1/40.
Cowpox G	Cowpox G	±	∓	—	—	—	—	—	—	—	—	—	—
	Vaccinia S	++++	+++	±	—	—	—	—	—	—	—	—	—
	nil	++++	++++	+++	++	∓	+++	+	∓	—	—	—	..
Vaccinia S	Cowpox G	+	∓	—	—	—	++++	+++	++	±	—	—	—
	Vaccinia S	++	±	—	—	—	+	—	—	—	—	—	—
	nil	++++	+++	+	—	—	++++	++++	+++	+	—	—	..
Normal rabbit	*nil*	—	—	..	..	..	—	—	..	..	..	..	..

Notations as in Table IV.

TABLE XI.—*Agglutination Tests with the Sera shown in Table X.*

Anti-sera.	Sera absorbed with E.Bs. of strain—	Elementary body suspensions. Cowpox G. Serum dilutions. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	Vaccinia S. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.
Cowpox G	Cowpox G	—	—	—	—	—	—	++	+	—	—	—	—
	Vaccinia S	++	++	+	±	—	—	++	++	±	—	—	—
	nil	+++	+++	+++	++	+	±	+++	+++	+++	+++	+	—
Vaccinia S	Cowpox G	—	—	—	—	—	—	+++	+++	+++	+++	+++	+
	Vaccinia S	—	—	—	—	—	—	++	+	—	—	—	—
	nil	+++	++	+	±	—	—	+++	+++	+++	+++	+++	+
Normal rabbit	*nil*	—	—	—	—	..	..	+	±	—	—	..	..

Notations as in Table V.

TABLE XII.—*Neutralization Tests with Sera shown in Table X.*

Anti-sera.	Sera absorbed with E.Bs. of strain—	Virus in serum-virus mixtures. Cowpox G. Dilutions of virus added to 1/5 dilution of sera. 10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	Vaccinia S. 10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	Serum alone.
Cowpox G	Cowpox G	27	25	24	12	6	..	24	21	16	15	12	..	—
	Vaccinia S	12	10	4	—	—	..	23	20	12	8	3	..	—
	nil	7	3	—	—	—	..	—	—	—	—	—	..	..
Vaccinia S	Cowpox G	21	27	26	14	4	..	17	—	—	—	—	..	—
	Vaccinia S	22	26	18	11	—	..	18	17	11	8	—	..	—
	nil	7	7	—	—	—	..	—	—	—	—	—	..	..
Normal rabbit	*nil*	31	23	25	14	5	—	21	21	16	15	14	—	..

Notations as in Table VI.

above. The results of the tests with the sera of cowpox strain G and vaccinia strain S are shown in Tables X, XI and XII.

It may be noted that the suspension of elementary bodies of vaccinia used for the agglutination test was slightly serum-sensitive, and the reactions with the lower dilutions of absorbed sera are therefore of doubtful significance. However, it is apparent from the complement-fixation and agglutination reactions with the unabsorbed sera that each serum shows a relatively higher titre with the homologous than with the heterologous antigen. This finding has been constant with different samples of immune sera, and suggests minor antigenic differences between the cowpox and vaccinia S strains. This difference in the unabsorbed sera is not apparent with the neutralization test where the serum concentration is kept constant and is relatively high. The results with the absorbed sera shown in the three tables indicate that the elementary bodies of each strain have absorbed the antibody for both viruses from its homologous serum and its own antibody from the heterologous serum, while only a partial reduction in titre for the other virus has been effected by absorption of the heterologous serum.

Similar results were obtained in corresponding cross-absorption tests with antisera to strains cowpox B and vaccinia S, although in this instance the results were not quite so sharp owing to the difficulty of absorbing the very potent sample of anticowpox B serum used. The results of the neutralization tests with the unabsorbed and absorbed antisera to cowpox B and vaccinia S are shown in Table XIII.

The amount of elementary bodies used to absorb these sera was obviously insufficient for complete absorption, and the suspension of vaccinia virus used for the serum-virus test mixtures had a relatively low titre. Nevertheless the results indicate the same relationship between cowpox strain B and vaccinia strain S as between cowpox strain G and vaccinia S.

DISCUSSION.

The results which have been obtained in cross-immunity experiments on recovered animals with cowpox and vaccinia strains and the results of neutralization tests with unabsorbed sera have confirmed the observations of other workers on the close immunological relationship between the virus of spontaneous cowpox and strains of vaccinia in current use. The closer analysis of the antigens of these viruses by means of absorption of hyperimmune sera with relatively pure suspensions of virus has, however, confirmed the impression gained from agglutination and complement-fixation tests with unabsorbed sera that, although similar, the antigens are not identical.

Serological tests with elementary body suspensions and Seitz filtrates of cowpox virus which had been heated to a temperature of 65° to 70° C. for 1 hour have indicated that this virus, like the vaccinia strains studied by Craigie and Wishart (1934*a* and 1936), and Parker and Rivers (1935), possesses heat-stable and heat-labile components ; but since a detailed examination of these antigens by means of pure " S " and pure " L " antisera has not been made, the difference between the cowpox and vaccinia strains has not been determined in relation to their heat-stable and heat-labile antigenic components.

TABLE XIII.—*Neutralization Tests with Antisera to Strains Cowpox B and Vaccinia S Unabsorbed and After Absorption with Elementary Bodies of the Two Strains.*

Anti-sera.	Sera absorbed with E.Bs. of strain—	Virus in serum-virus mixtures. Cowpox B. Dilutions of virus added to 1/5 dilution of sera. 10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	10^{-8}.	Vaccinia S. 10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	10^{-8}.	Serum alone.
Cowpox B	Cowpox B	17	16	19	12	4	..	11	12	5	—	—	..	—
	Vaccinia S	9	—	—	—	—	..	21	14	6	—	—	..	—
	nil	—	—	—	—	—	..	—	—	—	—	—	..	..
Vaccinia S	Cowpox B	24	22	22	17	5	..	—	—	—	—	—	..	—
	Vaccinia S	21	19	18	10	—	..	20	17	10	4	—	..	—
	nil	8	7	—	—	—	..	—	—	—	—	—	..	..
Normal rabbit	*nil*	22	22	21	20	14	9	20	22	20	16	—	—	..

Notations as in Table VI.

Strains of vaccinia virus, such as those in current use, can be produced by adaptation of smallpox virus to the rabbit, the cow or the sheep, but such a virus, as shown by the present work, differs from the virus of spontaneous cowpox. It seems likely that the adaptation of certain pock viruses to the rabbit as achieved by Gins (1919), Van Heelsbergen (1922) and others results in a modification of the antigens of the original strains towards a common structure, but, as Ledingham (1935) points out : " The mere fact that variola cowpox and horsepox can be made to throw off a type of variant possessing apparently similar properties—in fact, a sort of least common denominator which we designate vaccinia—in no way indicates identity of constituent antigen in the serological sense." The two strains of cowpox used in the above experiments have retained their original pathogenicity for the rabbit, and after 14 and 26 passages in this animal species extending over a period of 10 months show antigenic differences from a strain of vaccinia which had been adapted to this animal for a much longer period of time. With more prolonged propagation in the rabbit it is possible that the cowpox strains might become modified, so that the differences between them and the strains of vaccinia might disappear, but so far no evidence of such change has been obtained.

The opinion has been widely held that cowpox is due to infection from a human source, either from cases of variola or vaccinated individuals (see Gins, 1930). The epidemiological evidence, however, shows that, although the spread of infection in milch cows may be effected chiefly by the manipulations of the milkers, " spontaneous " cowpox may occur in cattle without any evidence of contact with variola or vaccinia in man (Frenkel, 1930 ; Blaxall, 1930). In this country cowpox appears to be much more prevalent in certain localities than others in spite of the widespread practice of vaccination of human beings against smallpox. Gins (1938) has recently stated that " für die Existenz einer den Rindern eigentümlichen und selbständigen Pockenkrankheit fehlt auch jetzt noch jeder Beweis ", but the histology of the lesions produced experimentally by strains of cowpox virus as recorded elsewhere (Downie, 1939) and the serological work reported in this paper show that the virus is different from the strains of vaccinia studied, and support the views of Ledingham (1935) and Findlay (1936) that cowpox is to be regarded as a disease *sui generis*.

SUMMARY.

The immunological relationship of two strains of the virus of spontaneous cowpox and two strains of vaccinia virus, one of which was known to have been derived from human variola, have been examined.

Rabbits recovered from infection of either cowpox virus or vaccinia were immune to both, and immune sera prepared against either virus neutralized both.

In agglutination and complement-fixation tests with hyperimmune sera the titres for the homologous virus were usually higher than for the heterologous virus.

By cross-absorption of such sera with elementary body suspensions of the viruses differences in their antigenic composition have been demonstrated.

The results of agglutination, complement-fixation and neutralization tests with the absorbed sera suggest that, although the antigens of cowpox and vaccinia are very much alike, there are qualitative differences in the heat-labile antigens.

These results and the differences in the lesions produced experimentally indicate that the virus of spontaneous cowpox is not the same as the strains of vaccinia virus examined.

REFERENCES.

BLAXALL, F. R.—(1930) ' System of Bacteriology ', Medical Research Council, Vol. VII, p. 151.

COPEMAN, S. M.—(1899) ' Vaccination : Its Natural History and Pathology ', London, Macmillan. Chap. ii, p. 41 ; chap. vi, p. 131.

CORY, R.—(1898) ' Lectures on the Theory and Practice of Vaccination ', London, Baillière, Tindall & Cox, p. 113.

CRAIGIE, J.—(1932) *Brit. J. exp. Path.*, **13**, 259.

Idem AND WISHART, F. O.—(1934*a*) *Brit. J. exp. Path.*, **15**, 390.—(1934*b*) *Trans. roy. Soc. Canada, Sect. V*, 91.—(1936) *J. exp. Med.*, **64**, 803 and 819.

CROOKSHANK, E. M.—(1889) ' History and Pathology of Vaccination ', London, H. K. Lewis & Co. Ltd., Vol. I, chap. ix, p. 287.

DAVIES, J. H. T., JANES, L. R., AND DOWNIE, A. W.—(1938) *Lancet*, ii, 1534.

DOWNIE, A. W.—(1939) *J. Path. Bact.*, **48,** 361.

FINDLAY, G. M.—(1936) *J. roy. micr. Soc.*, **56**, 213.

FRENKEL, H. S.—(1930) Eleventh int. vet. Cong., London, Vol. III, p. 22.

GINS, H. A.—(1919) *Z. Hyg. InfektKr.*, **89**, 231.—(1930) ' Handbuch der pathogenen Mikroorganismen ' (Kolle, Kraus and Uhlenhuth). Gustav Fischer, Jena. 3te Aufl. VIII, pp. 913, 917 and 976.—(1938) *Reichs-Gesundheitsblatt*, **13**, 201.

GLOVER, R. E.—(1937) Personal communication.

GREEN, A. B.—(1908) *Lancet*, ii, 719.

HORGAN, E. S.—(1938) *J. Hyg.*, **38**, 702.

JENNER, E.—(1798) ' An Inquiry into the causes and effects of the Variolæ Vaccinæ,' London. Republished 1896 by Cassell & Co.

LEDINGHAM, J. C. G.—(1935) *Proc. roy. Soc. Med.*, **29**, 73.

PARKER, R. F., AND RIVERS, T. M.—(1935) *J. exp. Med.*, **62**, 65.—(1936) *Ibid.*, **63**, 69.

REECE, R. J.—(1921) *Proc. roy. Soc. Med.*, **15**, Sect. Epidem., p. 13.

SALAMAN, M. H.—(1937) *Brit. J. exp. Path.*, **18**, 245.

VAN HEELSBERGEN, T.—(1922) *Zbl. Bakt.*, Abt. 1, **89**, 173.